MAJOR ARTICLE

Genetic Polymorphism in *Mycobacterium tuberculosis* Isolates from Patients with Chronic Multidrug-Resistant Tuberculosis

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Multidrug-resistant tuberculosis (MDR-TB) is a major public health problem because treatment is complicated, cure rates are well below those for drug-susceptible tuberculosis (TB), and patients may remain infectious for months or years, despite receiving the best available therapy. To gain a better understanding of MDR-TB, we characterized serial isolates recovered from 13 human immunodeficiency virus–negative patients with MDR-TB, by use of IS*6110* restriction fragment–length polymorphism analysis, spacer oligonucleotide genotyping (i.e., "spoligotyping"), and sequencing of *rpoB, katG, mabA-inhA* (including promoter), *pncA, embB, rpsL, rrs,* and *gyrA*. For all 13 patients, chronic MDR-TB was caused by a single strain of *Mycobacterium tuberculosis;* 8 (62%) of the 13 strains identified as the cause of MDR-TB belonged to the W-Beijing family. The sputum-derived isolates of 4 (31%) of the 13 patients had acquired additional drug-resistance mutations during the study. In these 4 patients, heterogeneous populations of bacilli with different resistance mutations, as well as mixtures of drug-susceptible and drug-resistant genotypes, were observed. This genetic heterogeneity may require treatment targeted at both drug-resistant and drug-susceptible phenotypes.

Multidrug-resistant tuberculosis (MDR-TB), which is caused by *Mycobacterium tuberculosis* isolates that are resistant to, at least, rifampin (RIF) and isoniazid (INH), is a serious public health hazard [1, 2]. Treating MDR-TB can be difficult, because loss of use of the 2 most potent anti-TB drugs (i.e., INH and RIF) means that only less effective "first-line" and "second-line" therapy, which is more toxic and less efficacious than INH and RIF, is available [3]. Thus, although, for some patients, MDR-TB can be cured by short-course chemotherapy [4–6], for other patients, bacillary growth is merely suppressed as long as treatment is continued [7]. Furthermore, 8%–35% of patients with MDR-TB have persistently active disease that is refractory to multidrug therapy [7–11]. Consequently, in most studies, the cure rates for MDR-TB remain well below those for drug-susceptible TB, and mortality rates may be substantial, even among HIV-negative patients [7]. In addition, patients with MDR-TB that does not respond to treatment are a constant source of transmission of multidrug-resistant *M. tuberculosis* [12–16].

In contrast to most bacteria, for *M. tuberculosis,* acquisition of drug resistance does not occur as a result of horizontal transfer of resistance-bearing genetic elements. Rather, acquisition of drug resistance by *M. tuberculosis* results from mutations (caused by nucleotide substitutions, insertions, or deletions) in specific resistance-determining regions of the genetic targets (or their promoters) or activating enzymes of anti-TB chemotherapeutic agents [17]. In *M. tuberculosis,* resis-

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Table 1. Characteristics of patients with multidrug-resistant (MDR) tuberculosis (TB).

Patient	Age, years	Sex	Previous TB	Duration of treatment, months[a]	MDR strain spoligotype[b]	Sputum AFB load[c]	Disease severity score[d]	No. of cultures[e]	Patient outcome status[e]
1	60	F	Y	30	34 (W321)	2.5	11.0	16	Alive
2	27	M	N	51	157	2.0	9.0	5	Deceased
3	29	F	N	28	34 (MH)	1.5	17.0	2	Deceased
4	32	M	Y	26	34 (W616)	3.0	13.5	4	Deceased
5	47	F	N	17	2	1.0	2.0	13	Alive
6	39	M	N	61	857	1.0	3.0	10	Alive
7	36	M	Y	69	2	1.5	14.5	11	Deceased
8	33	M	N	21	34 (W616)	3.0	15.5	6	Deceased
9	32	M	N	83	34 (W451)	2.0	8.0	4	Alive
10	25	F	Y	43	34 (W451)	1.5	10.0	2	Alive
11	44	F	N	41	79	1.0	13.0	8	Deceased
12	40	M	N	49	34 (W616)	2.5	11.0	7	Alive
13	22	M	Y	56	34 (W616)	2.5	15.0	11	Deceased

NOTE. AFB, acid-fast bacilli; spoligotype, spacer oligonucleotide genotype.

[a] No. of months of treatment before entry into the interferon-γ (i.e., present) study.

[b] Data are spoligotype (IS*6110* restriction fragment–length polymorphism profile).

[c] Sputum smears were recorded as having <4, 4–40, or >40 bacilli/high-power field, and they were given a score of 1, 2, or 3, respectively. Quantification was performed as described in Patients and Methods.

[d] As determined by chest radiography. Single lung cavities were given a score of 0.25, 0.5, or 1 point if they measured <2 cm, 2–4 cm, or >4 cm, respectively. Multiple lung cavities were given a score of 0.5, 1, or 2 points if they measured <2 cm, 2–4 cm, or >4 cm in diameter, respectively. Quantification was performed as described in Patients and Methods.

[e] During the study.

tance-conferring mutations occur spontaneously at a very low frequency. Inadequate therapy or subtherapeutic drug levels may provide a selective growth advantage and, thus, may favor the growth of a resistant phenotype that can ultimately predominate in persons in whom the disease was originally caused by drug-susceptible isolates [3]. Moreover, in patients with MDR-TB, selection for additional mutations may be accomplished by adding a single drug to a failing regimen [18].

In the human lung, selection of drug-resistance mutations in *M. tuberculosis* occurs predominantly within lung cavities for which high bacterial loads, active mycobacterial replication, and reduced exposure to host defense mechanisms have been reported [19–21]. Because *M. tuberculosis* in sputum samples obtained from patients with TB originates from lung cavities, molecular analysis of serially recovered sputum isolates allowed us to study aspects of the genetic evolution of drug resistance in the human host.

PATIENTS AND METHODS

Patients. We studied *M. tuberculosis* isolates recovered from 13 HIV-negative patients with MDR-TB that was refractory to chemotherapy given for $\geqslant$12 months. These 13 patients comprised the entire South African subset of a multinational, randomized, placebo-controlled study that evaluated the role of aerosolized recombinant human interferon-γ (rhuIFN-γ; InterMune) as an adjunct to chemotherapy for patients with MDR-TB [22]. Outcome analysis of this study of the use of rhuIFN-γ as an adjunct to antituberculous therapy is in progress and will be reported elsewhere. Consent was obtained from all patients studied, and the study was approved by the institutional review board of the University of Medicine and Dentistry of New Jersey (Newark) and the ethics committee of the University of Cape Town (Cape Town, South Africa).

All subjects were residents of the Western Cape region of South Africa, an area where TB is endemic (incidence of TB in 1999 was 546 cases/100,000 individuals). Because of a low prevalence of multidrug resistance in this area (0.3% in new patients and 1.7% in previously treated patients), all patients received treatment for drug-susceptible TB at diagnosis. Once our patients had MDR-TB diagnosed, drug regimens were individualized on the basis of the results of phenotypic drug-susceptibility tests. Drug susceptibility was evaluated every 2–3 months. For all patients, treatment regimens were adjusted, on the basis of the results of these evaluations, at ~6-month intervals. We performed a detailed microbiological analysis of the *M. tuberculosis* isolates recovered from these patients.

Sputum smears. Sputum smears obtained from all 13 patients remained positive for *M. tuberculosis* throughout the study. For each patient, the number of acid-fast bacilli present in sputum was determined, at entry into the interferon-γ (IFN-γ) study (i.e., at baseline), in 2 samples obtained separately (at least 2 weeks apart). By use of a standardized concentration procedure (incubation with 5% bleach, followed by high-speed centrifugation), smears of $\geqslant$2 cm^2 were stained with auramine and were examined using direct fluorescence microscopy with 400× magnification. Sputum smears were recorded as having

Table 2. Tuberculosis (TB) treatment received before and during the study.

	Initial TB treatment[a]		Treatment since diagnosis of multidrug-resistant TB[a]		Treatment during study[b]	
Patient	Drugs received	Duration, months	Drugs received	Duration, months	Drugs received	Duration, weeks
1[c]	R, H, Z, and E	12	R, H, Z, E, Et, Th, Sm, and K	18	E, O, and Cf	56
2	R, H, Z, and E	3	H, Z, E, Et, Th, Sm, K, O, Cf, and Te	48	E, Et, O, and Cf	12
3[c]	R, H, Z, and E	4	H, Z, E, Et, Th, A, K, O, Cf, Cy, and Cla	24	H, Th, A, O, Cf, and Cla	3
4[c]	R, H, Z, and E	8	H, Z, Th, K, and O	18	H, Z, Et, Th, and O	14
5	R, H, Z, and E	5	H, Z, E, Et, Th, Sm, and O	12	H, Z, E, Et, Th, and O	53
6[c]	R, H, Z, and E	5	H, Z, E, Et, Th, Sm, K, O, Cf, and Te	56	H, Z, E, Et, Th, O, Cf, and Te	52
7[c]	R, H, Z, and E	16	H, Z, E, Et, Th, Sm, K, O, and Cy	53	H, Z, E, Sm, and O	56
8	R, H, Z, and E	3	H, Z, E, Et, Th, Sm, K, O, and Cf	18	H, Z, E, Th, and O	30
9[c]	R, H, Z, and E	6	R, H, Z, E, Et, Th, Sm, K, O, and Cf	77	H, Z, E, Et, and Sm	28
10[c]	R, H, Z, and E	13	H, Z, E, Et, Th, Sm, and O	30	H, Z, E, Th, and O	8
11	R, H, Z, and E	7	R, H, Z, E, Et, Th, Sm, K, O, Cf, and Cla	34	H, Z, E, Et, Th, and O	17
12[c]	R, H, Z, and E	7	H, Z, E, K, and O	42	H, Z, E, and O	23
13	R, H, Z, and E	13	H, Z, E, Et, Th, Sm, K, O, and Cf	43	H, Z, E, Et, Th, and O	22

NOTE. A, amikacin; Cf, clofazimine; Cla, clarithromycin; Cy, cycloserine; E, ethambutol; Et, ethionamide; H, isoniazid; K, kanamycin; O, ofloxacin; R, rifampin; Sm, streptomycin; Te, terizidone; Th, thiacetazone; Z, pyrazinamide.

[a] Represents sequential events in the treatment history of each patient before entry into the interferon (IFN)–γ study.

[b] Represents the next sequential treatment and the duration of treatment received during the IFN-γ study from which sputum samples evaluated in the present study were obtained.

[c] Received aerosolized recombinant human IFN-γ (InterMune).

<4, 4–40, or >40 bacilli per high-power field, and they were given a score of 1, 2, or 3, respectively. For each patient, the mycobacterial load in sputum was calculated as the mean of the loads of 2 sputum specimens that were obtained 1 week apart and before administration of rhuIFN-γ and/or placebo.

Radiography. Chest radiographs were obtained at baseline and were analyzed to determine the extent of TB. Each lung was divided into 6 zones (upper [apex to second rib], middle [second to fourth rib], and lower zones [fourth rib to diaphragm]). Each zone was given a score of 0–2 points, according to the degree of pulmonary involvement: 0 denoted no involvement, 1 denoted that <50% of the lung was diseased, and 2 denoted that >50% of the lung was diseased. Single lung cavities were given a score of 0.25, 0.5, or 1 point if they measured <2 cm, 2–4 cm, or >4 cm, respectively. Multiple lung cavities were given a score of 0.5, 1, or 2 points if they measured <2 cm, 2–4 cm, or >4 cm in diameter, respectively. The disease severity score, as determined by evaluation of chest radiographs, thus ranged from 0 to 24 points [23].

Sputum cultures and drug-susceptibility testing. Sputum samples were processed in an automated mycobacterial culture system (MGIT; Becton Dickinson). Cultures positive for growth were examined by fluorescence microscopy, for the presence of acid-fast bacilli, and by polymerase chain reaction, to confirm the presence of *M. tuberculosis* [24]. The phenotypic resistance of all isolates was determined at baseline. Resistance to RIF and INH was confirmed by growth of all isolates in liquid culture medium that contained 2 μg/mL RIF or 0.1 μg/mL INH, by use of the radiometric Bactec 460 system (Becton Dickinson). The susceptibility of *M. tuberculosis* to several other antituberculous agents was determined using the indirect proportion method, by use of oleic acid, albumin, dextrose, catalase (OADC)–enriched solid 7H10 medium that contained critical concentrations of 7.5 μg/mL ethambutol (EMB), 10 μg/mL streptomycin, 5 μg/mL kanamycin, 2 μg/mL ofloxacin, 10 μg/mL ethionamide, or 2 μg/mL thiacetazone. Phenotypic susceptibility testing for pyrazinamide (PZA) was not performed, because the results of this test can be difficult to reproduce and may not correlate well with drug susceptibility in vivo [24, 25].

IS6110 restriction fragment–length polymorphism (RFLP) analysis. DNA RFLP analysis was performed on every *M. tuberculosis* isolate [26]. In brief, Loewenstein-Jensen slants were inoculated with 0.1 mL of MGIT broth and were incubated for 3–5 weeks. Mycobacterial DNA was extracted and was digested with *Pvu*II restriction endonuclease. DNA fragments were separated by SDS–PAGE, were Southern blot transferred, and were hybridized with a labeled IS*6110* probe. Bands were visualized by enhanced chemiluminescence and were analyzed with Whole Band Analyzer software (version 3.4; BioImage). Spacer oligonucleotide genotyping (i.e., "spoligotyping") was performed by amplification of the direct repeat region of each isolate. Isolates were assigned a number according to the Centers for Disease Control and Prevention nomenclature [27]. *M. tuberculosis* isolates that belonged to the W-Beijing family [28] were identified on the basis of high IS*6110* copy numbers, the presence

Table 3. Phenotypic and genotypic drug resistance in *Mycobacterium tuberculosis* isolates, as determined by cultures performed at baseline.

Patient	RIF	*RpoB*	INH	*katG*	*mabA-inhA*	PZA	*pncA*	EMB	*embB*	Sm	*rpsL, rrs*[a]	K	*rrs*	O	*gyrA*
1	R	D516V	**R**	***wt***[b]	***wt***	ND	96 (CAA ins)	S	*wt*[b]	S	*wt*	**S**	**A1401G**	S	*wt*[b]
2	R	S531L	R	S315T	*wt*	ND	*wt*[b]	**S**	**M306I**	**S**	**C492T, G217T *(rrs)***	S	G217T, C492T	R	A90V
3	R	L511R, D516V	R	S315T	G17T	ND	173 (G ins)	**S**	**M306I**	**S**	**A514C *(rrs)***	R	A1401G	R	D94G
4	R	S531L	R	*wt*	C15T	ND	Y103STOP	S	*wt*	R	K43R *(rpsL)*	R	C1402T	R	D94G
5	R	S531L	R	S315T	*wt*	ND	L85R	S	*wt*	S	*wt*	S	*wt*	S	*wt*
6	R	D516V	**R**	***wt***[b]	***wt***	ND	*wt*[b]	S	*wt*	**S**	**C492T *(rrs)***	S	*wt*	S	*wt*[b]
7	R	S531W	R	S315N	*wt*	ND	*wt*	**S**	**M306V**	S	*wt*	R	A1401G	R	A90V
8	R	S531L	R	*wt*	C15T	ND	Y103STOP	**S**	**M306V**	R	K43R *(rpsL)*	**S**	**A1401G**	S	*wt*
9	R	S531L	R	L101P	*wt*	ND	18 (24-bp del)	**S**	**M306V**	R	A514C *(rrs)*	S	*wt*	S	*wt*
10	R	S531L	**R**	***wt***	***wt***	ND	L27R	**S**	**M306I**	S	*wt*	S	*wt*	S	*wt*[b]
11	R	S531L	R	S315T	*wt*	ND	D8H	**S**	**M306I**	S	*wt*	S	*wt*	R	D94G
12	R	S531L	R	*wt*	C15T	ND	Y103STOP	**S**	**M306V**	R	K43R *(rpsL)*	S	*wt*	S	*wt*
13	R	H526Y	R	*wt*	C15T	ND	*wt*	S	*wt*	S	*wt*	**S**	**A1401G**	**S**	**A90V**

NOTE. Bold type denotes discordance between phenotypic and genotypic drug susceptibility. Genotypic resistance indicates amino acid substitution (for *rpoB, katG, embB, rpsL,* and *gyrA*) or nucleotide substitution *(inhA, pncA,* and *rrs).* del, Deletion; EMB, ethambutol; INH, isoniazid; ins, insertion; K, kanamycin; O, ofloxacin; PZA, pyrazinamide; R, resistant; RIF, rifampin; S, susceptible; Sm, streptomycin; *wt,* wild type.

[a] The gene in which the mutation was found is shown in parentheses.

[b] A resistance-conferring mutation was identified at a later time point (see table 4).

of characteristic IS*6110* insertions in the origin of replication region, and the presence of spoligotype S00034 [28, 29].

Genetic sequence analysis. Direct DNA sequencing of resistance-determining regions *(rpoB, katG, mabA-inhA* [including upstream regions], *pncA, embB, rpsL, rrs,* and *gyrA)* was performed on isolates obtained at baseline, during follow-up, and at the end of the study [17]. Whenever the sequence of ⩾1 gene had changed during the study, analysis of all isolates available was performed.

Statistical analysis. The 2-tailed Mann-Whitney *U* test was used to compare sputum bacterial loads and chest radiographic scores at baseline, and the 2-tailed *t* test was used to compare treatment durations. $P < .05$ was considered to be statistically significant.

RESULTS

Patients. Thirteen HIV-negative adults (8 men and 5 women; mean age, 35.8 years; age range, 22–60 years) were studied (table 1). All patients were sputum smear positive for MDR-TB and had received therapy for MDR-TB for a mean of 36 months (range, 12–77 months) at entry. Five patients had previously had TB, none of the patients had extrapulmonary TB, and 2 patients (patients 5 and 9) had diabetes mellitus; no other patients had specific risk factors for TB. None of the patients were related or had known epidemiologic links, and none were geographically clustered. Most patients excreted large numbers of bacilli in sputum (median score, 2.0) (table 1), and most patients had extensive disease noted on chest radiographs (median score, 11.0). Eight patients died during the 56 weeks of the study, most likely as a result of cachexia and/or chronic respiratory failure. Patients who died had more extensive disease (median score, 14.5), compared with patients who survived (median score, 9.0) ($P = .012$), despite these 2 groups of patients having had a similar mean duration of disease (41.7 vs. 47.2 months; $P = .64$) (table 1). A mean of 7.6 cultures/patient (range, 2–16 cultures/patient) was available for analysis.

At the time that TB was originally diagnosed, all patients were treated with World Health Organization (WHO) category I therapy (i.e., treatment with INH, RIF, PZA, and EMB for 2 months, followed by treatment with either INH and RIF or INH, RIF, and PZA for an additional 4 months) for varying lengths of time (table 2). Once MDR-TB was diagnosed, the patients were switched to treatment regimens tailored to the phenotypic drug-susceptibility profile of their isolates (table 2). At entry to the study, therapy was again adjusted according to phenotypic drug susceptibility, treatment history, and the side-effect profile. Eight of 13 patients received aerosolized rhuIFN-γ as an adjunct to treatment (table 2).

***Phenotypic and genotypic resistance profile of* M. tuberculosis.** The phenotypic and genotypic drug resistance of each patient is shown in table 3. All isolates displayed phenotypic resistance to INH. Eight isolates had single-nucleotide substitutions in *katG,* and 4 harbored the C15T mutation in the promoter region of *mabA-inhA*. In 1 INH-resistant strain (in patient 10), neither *katG* nor *mabA-inhA* mutations were identified (table 3). Eleven patients continued receiving INH, despite having documented resistance to this drug. Eight of these 11 patients received thiacetazone, which was only available in a combination that contained INH. For all isolates, resistance to RIF was confirmed by phenotypic and genotypic *(rpoB)* resistance testing. Phenotypic resistance to EMB was not observed in any of the 13 isolates. However, sequence analysis

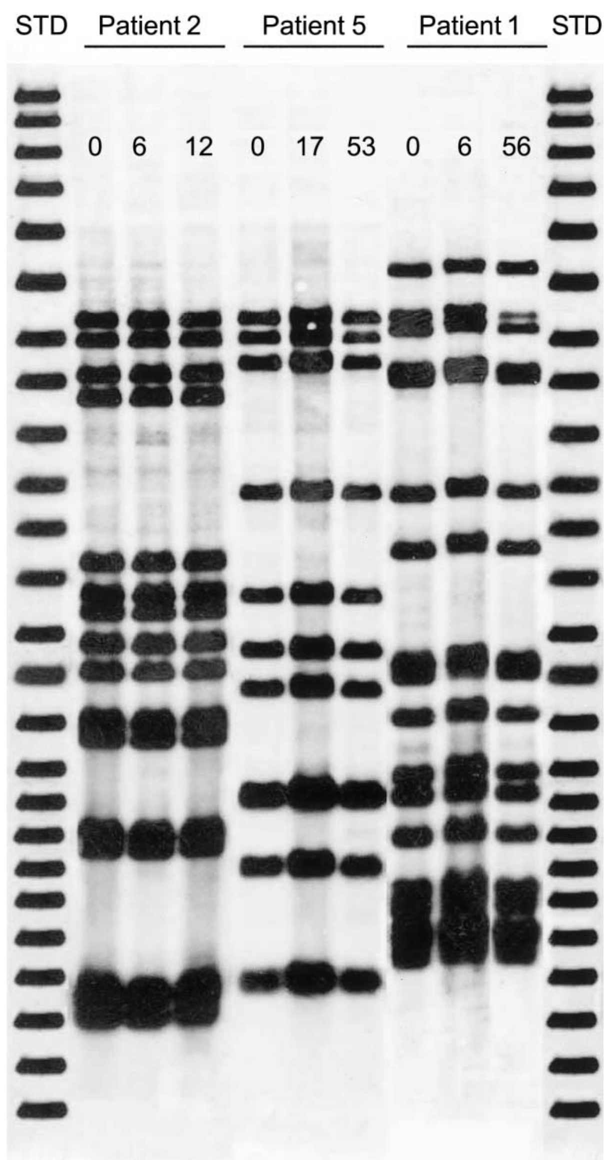

Figure 1. IS*6110* restriction fragment–length polymorphism profiles of *Mycobacterium tuberculosis* isolated from sputum samples obtained from 3 patients at baseline (0) and after various lengths of treatment (6, 12, 17, 53, or 56 weeks). STD, molecular weight standard.

of *embB,* the genetic target of EMB, revealed resistance mutations in 62% of isolates. The presence of aminoglycoside resistance mutations in *rrs* was poorly predicted by the results of phenotypic susceptibility tests. Taken together, these results suggest that the isolates from all 13 patients were highly resistant to many of the most potent first- and second-line agents.

Genetic analysis of serially recovered sputum isolates. Despite living in an area with high rates of transmission of *M. tuberculosis,* the 13 patients who were studied had no evidence of coinfection with a second *M. tuberculosis* strain. This was demonstrated by the fact that all *M. tuberculosis* isolates recovered from the same patient showed identical IS*6110* RFLP profiles (figure 1) and spoligotype patterns. It is of interest to note that 8 (62%) of 13 isolates recovered from the patients with treatment-refractory MDR-TB belonged to either the W-Beijing family ($n = 7$) or the W-Beijing lineage ($n = 1$) [29]. Of note, patients who were infected with W-Beijing isolates had higher sputum bacterial loads (median bacillary load, 2.5) than did patients who were infected with non–W-Beijing isolates (median bacillary load, 1.0) ($P = .019$).

Sequence analysis of serially recovered* M. tuberculosis *sputum isolates. *M. tuberculosis* drug-resistance polymorphism was explored by sequencing the genes of serially recovered isolates (2–3 isolates/patient). For 9 patients, all drug targets that were examined remained unchanged, from baseline, during the 3–56-week study (table 3). However, for 4 patients (31%), additional drug-resistance mutations had been selected during treatment (table 4). For patient 1, polymorphisms were noted in *katG, embB,* and *gyrA*. The *gyrA* gene mutation was found in some, but not all, bacilli at multiple time points. Patient 2 had polymorphisms in *pncA, gyrA,* and *rrs;* the *pncA* gene was mutated in some, but not all, bacilli at 2 time points. Patient 6 had polymorphisms in *gyrA, pncA, rpoB,* and *katG,* and the *gyrA* gene was mutated in some, but not all, bacilli at 1 time point. In patient 10, both *gyrA* and *pncA* had polymorphisms, and the *pncA* gene was mutated in some, but not all, bacilli isolated at 1 time point. For 3 of these patients, different nucleotide substitutions accounted for drug resistance in bacillary subpopulations (*gyrA* D94G, D94Y, and G94C accounted for resistance to fluoroquinolone in patient 1; *gyrA* A90V and D94Y accounted for resistance in patient 2; and *rpoB* D516V and S531W accounted for resistance to rifampin in patient 6; both the *pncA* V157G substitution and the 131GG insertion accounted for resistance to PZA in patient 6) (table 4). As seen in patients 1 and 6, isolates that harbored the wild-type *(wt) katG* gene (and, thus, potentially were susceptible to INH) were still recovered several years after the patient had developed MDR-TB (table 4).

DISCUSSION

Our studies show that the selection of drug resistance (a stochastic event) during treatment of TB results in a mixture of subpopulations of bacilli, some of which are drug resistant, whereas others are either drug susceptible or bear different genetic mutations that account for the resistance to the same drug (table 4). In the presence of anti-TB therapy, drug-resistant bacilli would be expected to outgrow drug-susceptible bacilli over time. The observed mixture of susceptible and resistant organisms suggests that the mutations may have been acquired recently. Polyclonal and heteroresistant populations of bacilli in sputum have been reported elsewhere [30–34]. In the present study, we demonstrate the presence of clonal discrete founder strains (on the basis of IS*6110* RFLP analysis)

Table 4. Genotypic polymorphism of selected *Mycobacterium tuberculosis* genes in 4 patients.

Week of therapy	Patient 1			Patient 2			Patient 6				Patient 10	
	KatG	*embB*	*gyrA*	*pncA*	*gyrA*	*rrs*	*gyrA*	*pncA*	*rpoB*	*katG*	*gyrA*	*pncA*
0	*wt*	*wt*	*wt*	*wt*	A90V	G217T, C492T	*wt*	*wt*	D516V	*wt*	*wt*	L27R/*wt*
1	*wt*	*wt*	*wt*	…	…	…	…	…	…	…	…	…
2	*wt*	*wt*	*wt*	**D12A/*wt***	**D94Y**	**C492T**	…	…	…	…	…	…
4	**G118A**	*wt*	*wt*	…	…	…	…	…	…	…	…	…
6	G118A	**G406D**	**D94Y/*wt***	**D12A**	D94Y	C492T	*wt*	*wt*	**S531W**	**E261K**	…	…
8	***wt***	G406D	D94Y/*wt*	**D12A/*wt***	D94Y	C492T	*wt*	*wt*	S531W	E261K	**D94G**	***wt***
12	*wt*	G406D	D94Y/*wt*	**D12A**	D94Y	C492T	…	…	…	…	…	…
16	*wt*	G406D	D94Y/*wt*	…	…	…	*wt*	*wt*	S531W	E261K	…	…
24	*wt*	G406D	D94Y/*wt*	…	…	…	…	…	…	…	…	…
30	*wt*	G406D	**D94G**	…	…	…	*wt*	*wt*	S531W	***wt***	…	…
36	*wt*	G406D	**D94Y**	…	…	…	**G88A**	**V157G**	S531W	**E261K**	…	…
40	*wt*	G406D	**D94C/*wt***	…	…	…	***wt***	**131 (GG ins)**	S531W	E261K	…	…
44	*wt*	G406D	D94C/*wt*	…	…	…	**G88A/*wt***	131 (GG ins)	S531W	E261K	…	…
48	*wt*	G406D	D94C/*wt*	…	…	…	***wt***	131 (GG ins)	S531W	E261K	…	…
52	*wt*	G406D	**D94G**	…	…	…	*wt*	131 (GG ins)	S531W	E261K	…	…
56	*wt*	G406D	D94G	…	…	…	…	…	…	…	…	…

NOTE. Bold type denotes changes in genotype. ins, Insertion; *wt,* wild type.

that are composed of heterogeneous populations of bacilli that differ in their resistance profile. In a recent ex vivo study of multiple lesions obtained from excised human lungs, we noted that, for 3 of 3 cases, discrete founder strains of *M. tuberculosis* acquired resistance independently in separate physical locales within the same lung. This finding suggested the occurrence of parallel evolution—that is, the independent emergence of different bacillary genotypes within the same lung [21]. Sputum samples obtained from such a patient would likely contain bacillary subpopulations with a mixture of drug-resistant genotypes, similar to what was observed in the present study.

Phenotypic drug resistance is commonly determined by the proportion method, in which an *M. tuberculosis* isolate is considered to be resistant to a drug if $\geqslant$1% of colonies grow on agar that contains a critical concentration of that drug [35]. Consequently, the particular drug can be withdrawn or withheld [3, 36]. Our observations suggest that it may not be prudent to withdraw the drug(s) after documentation of drug resistance. Rather, as is often practiced in the field, the existing drug(s) should be maintained to target the bacilli that are still drug susceptible. In addition, new drugs (preferably, $\geqslant$2) should be added to the treatment regimen to target the newly drug-resistant organisms. For example, improved outcomes for patients with MDR-TB who were receiving therapy involving INH together with the other drugs have been reported [13].

In accordance with the findings of previous studies, acquisition of drug-resistance mutations was not associated with changes in the IS*6110* profile [21, 37, 38]. During the course of our study, the IS*6110* patterns of all 13 *M. tuberculosis* isolates did not change (7.2 person-years of observation), nor was any super infection detected. This was the case despite the high disease burden and rates of transmission of TB in the Western Cape and despite what appears to be constant exposure to TB in the environment [39]. In most published studies, evolutionary changes in IS*6110* patterns occurred in <10% of patients, and it has been suggested that these events might be more frequent soon after infection, when replication is most active [37, 40, 41]. The small number of patients in our study and the long periods of active disease before enrollment may have contributed to the lack of any IS*6110* instability in the present study.

Of note, 62% of cases of multidrug-resistant disease in our sample were caused by *M. tuberculosis* strains that belonged to the W-Beijing family of strains (spoligotype 34) (table 1) [28]. These strains have been reported to comprise ~20% of all *M. tuberculosis* isolates in the Western Cape [42]. The relatively high frequency of W-Beijing strains seen in our study may simply reflect a sampling bias rather than a significant difference between W-Beijing strains and other strains [43]. However, it may also reflect a specific predilection of this family of strains for developing drug resistance, as has been suggested elsewhere [44–46]. Alternatively, multidrug-resistant W-Beijing strains may have a comparatively higher propensity to spread in the community. Our observation of higher bacillary loads, at baseline, in the sputum of patients infected with W-Beijing strains may support this notion (table 1).

In agreement with previous studies, we observed a good correlation between phenotypic and genotypic susceptibility to INH and RIF [47–49]. The single patient in whom no *katG* or *mabA-inhA* mutations were identified may have had mutations in *kasA, ahpC* [17], or the recently identified INH target gene *ndh,* which encodes the enzyme NADH dehydrogenase, none

of which was investigated in this study [50]. In contrast, the phenotypic and genotypic susceptibility to EMB correlated poorly [51, 52]. Together with nonavailability of susceptibility data for PZA (table 3), overestimation of the number of active drugs used to treat most of the patients in our study may have occurred [53].

The results of the present study, together with our previous analysis of multiple lesions in lung samples obtained from patients with chronic progressive TB [21], suggest that, in designing therapeutic strategies, we need to consider that the sputum used for diagnosis and for drug-susceptibility testing represents a static single sampling of a dynamic process. That is, the organisms found in a single sputum sample may not be representative of the entire population of organisms in the affected lung, as well as in other infected sites. New and improved diagnostic and drug-susceptibility testing methods that can detect heterogeneous subpopulations need to be developed to improve treatment of MDR-TB.

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